JOURNAL OF BACTERIOLOGY, July 2006, p. 4727–4736 Vol. 188, No. 13
0021-9193/06/$08.00+0 doi:10.1128/JB.00167-06

YtsJ Has the Major Physiological Role of the Four Paralogous Malic Enzyme Isoforms in *Bacillus subtilis*†

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Received 31 January 2006/Accepted 16 April 2006

The *Bacillus subtilis* genome contains several sets of paralogs. An extreme case is the four putative malic enzyme genes *maeA*, *malS*, *ytsJ*, and *mleA*. *maeA* was demonstrated to encode malic enzyme activity, to be inducible by malate, but also to be dispensable for growth on malate. We report systematic experiments to test whether these four genes ensure backup or cover different functions. Analysis of single- and multiple-mutant strains demonstrated that *ytsJ* has a major physiological role in malate utilization for which none of the other three genes could compensate. In contrast, *maeA*, *malS*, and *mleA* had distinct roles in malate utilization for which they could compensate one another. The four proteins exhibited malic enzyme activity; MalS, MleA, and MaeA exhibited 4- to 90-fold higher activities with NAD^+ than with $NADP^+$. YtsJ activity, in contrast, was 70-fold higher with $NADP^+$ than with NAD^+, with K_m values of 0.055 and 2.8 mM, respectively. *lacZ* fusions revealed strong transcription of *ytsJ*, twofold higher in malate than in glucose medium, but weak transcription of *malS* and *mleA*. In contrast, *mleA* was strongly transcribed in complex medium. Metabolic flux analysis confirmed the major role of YtsJ in malate-to-pyruvate interconversion. While overexpression of the NADP-dependent *Escherichia coli* malic enzyme MaeB did not suppress the growth defect of a *ytsJ* mutant on malate, overexpression of the transhydrogenase UdhA from *E. coli* partially suppressed it. These results suggest an additional physiological role of YtsJ beyond that of malate-to-pyruvate conversion.

The phosphoenolpyruvate (PEP)-pyruvate-oxaloacetate (OAA) node is a key hub in central carbon metabolism, linking glycolysis and the Krebs cycle (Fig. 1). The metabolite interconversions at this level require a set of structurally complex reactions that link the principal pathways of carbon metabolism and thus regulate the carbon fluxes among catabolism, anabolism, and the energy needs of cells (30). Gluconeogenic and anaplerotic reactions in and out of the Krebs cycle require C_3 (PEP and/or pyruvate) carboxylations and C_4 (malate and/or OAA) decarboxylations. Among these, the malic enzymes catalyze the decarboxylation of malate to pyruvate, with concomitant reduction of $NAD(P)^+$. Several malic enzyme isoforms have been characterized in eukaryotes, which are grouped into three classes according to their coenzyme specificity and ability to decarboxylate OAA (23). Enzymes of the first class (EC 1.1.1.40) are NADP dependent and can decarboxylate both malate and OAA; they are found in the cytosol, in chloroplasts, and in mitochondria. Enzymes of the second class (EC 1.1.1.38) preferentially utilize NAD and are also capable of decarboxylating OAA. Those of the third class (EC 1.1.1.39) are NAD-dependent malic enzymes that are unable to use OAA and are exclusively located in the mitochondrial matrix.

The ubiquitous distribution of malic enzymes highlights their important biological functions, which include carbon fixation in tropical C_4 plants, lipogenic NADPH production in fungi and animals, and energy production in rapidly proliferating tissues by the mitochondrial isoform (9). In prokaryotes, malic enzymes are also widely distributed but few are well characterized. In *Escherichia coli*, both NAD- and NADP-dependent malic enzymes, SfcA and MaeB, respectively, have been studied in detail (27, 19, 20, 31). Their respective physiological functions have not been precisely determined, but it has been suggested that SfcA is involved in gluconeogenesis by providing pyruvate (which is then converted to PEP by a PEP synthase) (20) and that MaeB is involved in the supply of NADPH and acetyl coenzyme A, which is necessary for anabolism reactions during growth on C_4 dicarboxylates as sole carbon sources (27, 19, 20). SfcA synthesis is inducible by malate, and the synthesis of both enzymes has been found to be repressed in the presence of glucose (27). Combined deletion of the two genes leads to a severe growth defect only on C_4 dicarboxylates, thus indicating that malic enzyme activity is necessary under such growth conditions in *E. coli* (33). *Rhizobium meliloti*, a nitrogen-fixing gram-negative symbiote of alfalfa, also synthesizes two malic enzymes named DME and TME, which are NAD and NADP dependent, respectively (12, 26). The former has been shown to be involved in symbiotic nitrogen fixation as a result of its role in growth in the nodule, where C_4 dicarboxylates are the major carbon and energy source provided by the host (10, 13), while the latter has been proposed to function as a generator of NADPH necessary for

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† Supplemental material for this article may be found at http://jb.asm.org/.

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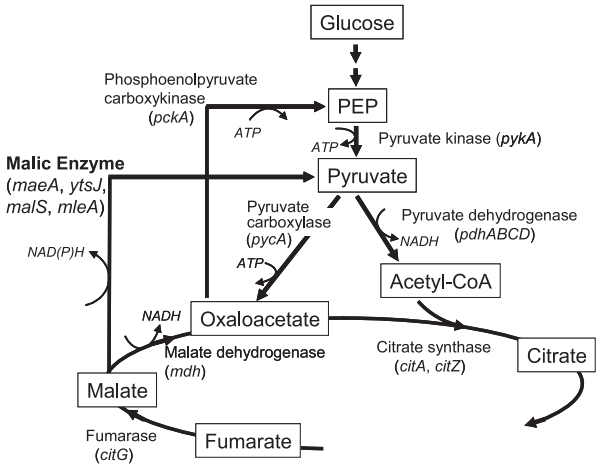

FIG. 1. The PEP-pyruvate-OAA node in *B. subtilis*. Acetyl-CoA, acetyl coenzyme A.

biosynthetic reactions (11). Thus, in bacteria, malic enzymes seem to play the same roles as in higher organisms: NAD(P)-dependent malic enzymes, together with pyruvate dehydrogenase, provide acetyl coenzyme A from malate; the NADP-dependent ones generate NADPH for anabolism (17).

Bioinformatic analysis of the *Bacillus subtilis* genome has revealed the presence of four paralogous genes encoding putative malic enzymes: *maeA* (formerly *ywkA*), *malS*, *ytsJ*, and *mleA*. On the basis of protein sequence comparisons, these putative malic enzymes can be distributed into two groups, MaeA and MalS on the one hand and YtsJ and MleA on the other (8). In a previous study, we have demonstrated that MaeA possesses malic enzyme activity and exhibits a clear preference for NAD^+ as a coenzyme. We have also shown that expression of *maeA* is induced by the presence of malate. This transcriptional regulation is mediated by the two-component system MalK-MalR (formerly YufL-YufM) (8). These results confirmed the original observation of the stimulation of the synthesis of an NADP/NAD-dependent malic enzyme activity in the presence of malate (7). Furthermore, the two-component system MalK-MalR has been shown to control the malate-dependent expression of two other genes, *maeN* and *yflS*, encoding malate transporters (32). No information is available concerning the other three putative malic enzyme genes, except that *mleA* has been predicted to encode a malolactic rather than a malic enzyme, and thus to catalyze the conversion of malate into lactate, on the basis of its putative cotranscription with *mleN*, which encodes a malate-lactate antiporter (34). The two malolactic enzymes from lactic acid bacteria (*Lactococcus lactis* and *Oenococcus oeni*) characterized to date clearly belong to the malic enzyme family (4).

The current study was designed to characterize the respective function of each putative malic enzyme gene of *B. subtilis*. The expression pattern of these genes was analyzed; the corresponding gene products were purified, and their enzymatic activities were characterized. To investigate the physiological role of these genes, single- and multiple-mutant strains were constructed and their phenotypes were studied under different growth conditions. These experiments have revealed a major and complex physiological role for *ytsJ*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* TG1 [K12 Δ(*lac-pro*) *supE thi hsd5*/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*Δ*M15*] was used as a general cloning host, and *E. coli* strain M15(pREP4) (QIAGEN) was used for overproduction of His_6-MalS, His_6-YtsJ, His_6-MleA, and His_6-MaeA. *E. coli* strains were grown in Luria-Bertani broth (LB) supplemented with antibiotics when necessary (ampicillin,100 mg/liter; kanamycin, 25 mg/liter). A conventional calcium shock procedure was used for transformation (28).

The *B. subtilis* strains used in this work are listed in Table 1. Standard procedures were used to transform *B. subtilis* (1). *B. subtilis* strains were grown in LB except when stated otherwise. Antibiotics for selection were added at 5 mg/liter (chloramphenicol), 0.4 mg/liter (erythromycin), 100 mg/liter (spectinomycin), 5 mg/liter (kanamycin), or 0.2 mg/liter (phleomycin). Cultures were performed at 37°C. Growth tests were performed in C minimal medium (2) [K_2HPO_4 at 70 mM, KH_2PO_4 at 30 mM, $(NH_4)_2SO_4$ at 25 mM, $MgSO_4$ at 0.5 mM, $MnSO_4$ at 0.01 mM, ferric ammonium citrate at 22 mg/liter] supplemented with tryptophan at 50 mg/liter and carbon sources (10 g/liter). The cultures were inoculated from precultures in the same medium (excepted for GM1622, which was precultured in C glucose). Cultures for β-galactosidase assays were performed in CQTHC minimal medium (C minimal medium supplemented with tryptophan 50 at mg/liter, glutamine at 0.5 g/liter, and casein hydrolysate at 0.5 g/liter) supplemented with carbon sources (10 g/liter).

DNA manipulation. PCR products were purified with the QIAquick PCR purification kit (QIAGEN). Plasmid DNA was extracted and purified from *E. coli* with the QIAprep spin miniprep kit (QIAGEN). Restriction enzymes and T4 DNA ligase were used as recommended by the manufacturers. *Pfu* DNA polymerase (Invitrogen) was used for preparative PCRs as recommended by the manufacturer. DNA fragments were purified from agarose gels with the QIAGEN gel extraction kit.

Strain construction. Fusions of *ytsJ*, *malS*, *ytnP*, and *mleN* promoter fragments to a promoterless *lacZ* gene were constructed with the vector pDG1661 (18). The different promoter fragments to be tested were obtained by PCR with primers GLO03 and GLO04 for *ytsJ*, GLO06 and GLO07 for *malS*, GLO05 and GLO07 for *ytnP*, and GLO08 and GLO09 for *mleN* (Table S1 in the supplemental material). These fragments were inserted between the EcoRI and BamHI sites of pDG1661 to generate plasmids pGL00, pGL01, pGL02, and pGL03, respectively. These plasmids were used to insert the reporter constructs into the *B. subtilis amyE* locus to generate strains GM1600, GM1601, GM1602, and GM1603, respectively (Table 1). Comparisons with *lacZ* fusions at the homologous locus (for *ytsJ* and *ytnP-malS*) indicated that the promoter fragments designed for construction of strains GM1600 and GM1602 contain all of the *cis*-acting elements for regulated expression of *ytsJ* and *ytnP*, respectively; this was confirmed by disruption tests and reverse transcription-PCR analyses for the *ytsJ* promoter-containing fragment (data not shown). The *mleN-mleA* promoter fragment used contains the 260 bp preceding the *mleN* start codon; it thus includes the last 126 bp of the upstream *asnB* gene and the transcription terminator of the *asnA-asnB* operon.

Strain GM1608 contains a disruption of the *ytsJ* open reading frame (ORF) obtained by the insertion of a pMUTIN derivative plasmid containing an internal fragment of the *ytsJ* ORF (pHV30253). The in vivo replacement by homologous recombination of a kanamycin resistance cassette for the *lacZ* and erythromycin resistance genes of pMUTIN was then obtained with pEC23, a plasmid specifically designed for this purpose and constructed by P. Stragier. Strain GM1632 was constructed by interruption of the *mleA* ORF by simple crossover integration of pGL12, a derivative of vector pJH101 (14) containing an internal fragment of the *mleA* ORF.

β-Galactosidase assays. β-Galactosidase activities were measured by the method of Miller (25) on cell extracts prepared by lysozyme treatment and centrifugation. Protein concentration was determined by the Bradford method (3) with the Bio-Rad protein assay solution (Bio-Rad). One unit of β-galactosidase activity is defined as the amount of enzyme that produces 1 nmol of *o*-nitrophenol/min at 20°C. No endogenous β-galactosidase activity in parental strain 168CA, which is known to express β-galactosidase at low levels only during sporulation (5), was detected under the conditions used here.

Expression and purification of His_6-tagged proteins. His_6-tagged proteins were overexpressed and purified with the QIAexpress kit (QIAGEN). *ytsJ*, *malS*, and *mleA* coding sequences were integrated into *E. coli* expression vector pQE-30 with corresponding primers (Table S1 in the supplemental material) in order to produce fusion proteins with six histidines at the N terminus. The resulting plasmids, pEC05, pEC06, and pEC07, respectively, were transformed into *E. coli* strain M15(pREP4) to generate the strains used for production. *E. coli* strain GTDp4, previously constructed in our laboratory (8), allowed over-

TABLE 1. *Bacillus subtilis* strains used in this study

Strain	Relevant genotype	Source or reference
168CA	*trpC2*	Laboratory stock
BFA91	*ytsJ′*::pMUTIN2 (*ery*)	BSFA[a] collection
GM1608	*ytsJ′*::[pMUTIN2 Δ(*lacZ-ery*)::*kan*]	pEC23 → BFA91
GTD102	*maeA′*::*kan*	8
GTD110	Δ*malS*::*spc*	8
GM1632	*mleA′*::*cat*	This work
GTD111	*maeA′*::*kan* Δ*malS*::*spc*	8
GTD121	*ytsJ′*::pMUTIN2 (*ery*) *maeA′*::*kan*	8
GTD122	*ytsJ′*::pMUTIN2 (*ery*) Δ*malS*::*spc*	8
GM1645	*ytsJ′*::[pMUTIN2 Δ(*lacZ-ery*)::*kan*] *mleA′*::*cat*	GM1632 DNA × GM1608
GTD123	*ytsJ′*::pMUTIN2 (*ery*) *maeA′*::*kan* Δ*malS*::*spc*	GTD110 DNA × GTD121
GM1655	*maeA′*::*kan* Δ*malS*::*spc mleA′*::*cat*	GM1632 DNA × GTD121
GM1621	*ytsJ′*::pMUTIN2 (*ery*) *maeA′*::*kan* Δ*malS*::*spc mleA′*::*cat*	GM1632 DNA × GTD123
SJB238	*mdh′*::*spc*	22
GM1614	*mdh′*::*spc*	SJB238 DNA × 168CA
GM1622	*ytsJ′*::pMUTIN2 (*ery*) *maeA′*::*kan* Δ*malS*::*spc mleA′*::*cat mdh′*::*spc*	GM1614 DNA × GM1621
PS1716	*amyE′*::P*maeA′*::*lacZ-cat*	8
GM1600	*amyE′*::P*ytsJ′*::*lacZ-cat*	pGL00 → 168CA
GM1601	*amyE′*::P*malS′*::*lacZ-cat*	pGL01 → 168CA
GM1602	*amyE′*::P*ytnP′*::*lacZ-cat*	pGL02 → 168CA
GM1603	*amyE′*::P*mleN′*::*lacZ-cat*	pGL03 → 168CA
GM1658	*ytsJ′*::[pMUTIN2 Δ(*lacZ-ery*)::*kan*]/pND23	pND23 → GM1608
GM1659	*trpC2*/pND23	pND23 → 168CA
GM1660	*ytsJ′*::[pMUTIN2 Δ(*lacZ-ery*)::*kan*]/pGL04 (*ytsJ*)	pGL04 → GM1608
GM1661	*ytsJ′*::[pMUTIN2 Δ(*lacZ-ery*)::*kan*]/pGL05 (*malS*)	pGL05 → GM1608
GM1662	*ytsJ′*::[pMUTIN2 Δ(*lacZ-ery*)::*kan*]/pGL06 (*mleA*)	pGL06 → GM1608
GM1663	*ytsJ′*::[pMUTIN2 Δ(*lacZ-ery*)::*kan*]/pGL07 (*maeA*)	pGL07 → GM1608
GM1664	*ytsJ′*::[pMUTIN2 Δ(*lacZ-ery*)::*kan*]/pGL08 (*sfcA*)	pGL08 → GM1608
GM1665	*ytsJ′*::[pMUTIN2 Δ(*lacZ-ery*)::*kan*]/pGL09 (*maeB*)	pGL09 → GM1608
GM1666	*ytsJ′*::[pMUTIN2 Δ(*lacZ-ery*)::*kan*]/pGL10 (*udhA*)	pGL10 → GM1608
GM1667	*ytsJ′*::[pMUTIN2 Δ(*lacZ-ery*)::*kan*]/pGL11 (*pntAB*)	pGL11 → GM1608
GM1669	*ytsJ′*::[pMUTIN2 Δ(*lacZ-ery*)::*kan*]/pGL16 (*maeBΔ1*)	pGL16 → GM1608
GM1670	*ytsJ′*::[pMUTIN2 Δ(*lacZ-ery*)::*kan*]/pGL17 (*maeBΔ2*)	pGL17 → GM1608

[a] BSFA, Systematic Function Analysis of *Bacillus subtilis* Genes Project.

expression of His_6-MaeA. All of the following procedures were performed as described in the QIAexpress manual. His_6-tagged proteins were stored in a buffer containing Tris at 50 mM (pH 8), NaCl at 150 mM, dithiothreitol at 1 mM, and EDTA at 0.5 mM.

Determination of kinetic parameters of YtsJ, MalS, and MleA. YtsJ, MalS, and MleA malic enzyme activities were tested at 37°C by spectrophotometrically monitoring, at 340 nm, NAD(P)H formation during the reductive decarboxylation of malate [malate plus NAD(P) into pyruvate plus NAD(P)H]. The reaction mixture was composed of 25 nmol of protein in 1 ml of buffer containing divalent cations (Tris-HCl at 50 mM [pH 8], $MgCl_2$ at 10 mM, $MnCl_2$ at 10 mM, KCl at 50 mM, β-mercaptoethanol at 10 mM). For determination of the K_m for malate, both cofactors, NAD^+ and $NADP^+$, were added to the reaction mixture at 5 mM. For determination of the K_m for each cofactor, the malate concentration was raised to 40 mM. For both cofactors, a molar extinction coefficient of 6.22×10^6 cm^2 mol^{-1} was used for calculations. The catalytic constant (k_{cat}) is expressed per subunit. The initial rate data of the reaction were fitted to the Michaelis-Menten relationship by least-squares analysis to determine K_m and k_{cat}. Demonstration of malic enzyme activity was assessed in a coupled reaction: at the end of the first reaction (end of NAD reduction), 40 U of strictly NAD-dependent lactate dehydrogenase from *Bacillus stearothermophilus* (Sigma) and 10 mM $CaCl_2$ were added to the reaction mixture. The oxidation of NADH, if produced by the first conversion of malate to pyruvate, was then monitored. Demonstration of the ability of each protein to decarboxylate OAA was realized by monitoring spectrophotometrically, at 430 nm, the disappearance of OAA after addition of YtsJ, MalS, MleA, or MaeA (1 U is defined as the activity leading to a decrease of 1 U of optical density at 430 nm [OD_{430}]/min).

Overexpression of homologous and heterologous malic enzymes and transhydrogenase systems in *B. subtilis*. The *ytsJ*, *maeA*, *malS*, *mleA*, *sfcA*, *maeB*, *udhA*, and *pntAB* genes were overexpressed in vivo in the GM1608 strain. The ORF of each gene has been amplified by PCR with corresponding primers and inserted into the pND23 replicative expression vector (6) downstream of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter and an optimized ribosome binding site, between the BamHI and SphI sites for *ytsJ* and *malS*, between the BamHI and XhoI sites for *udhA*, between the BamHI and EcoRI sites for *maeB*, and between the HindIII and BamHI sites for *maeA*, *mleA*, *sfcA*, and *pntAB*. Plasmids pGL04, pGL05, pGL06, pGL07, pGL08, pGL09, pGL10, and pGL11, containing the *ytsJ*, *malS*, *mleA*, *maeA*, *sfcA*, *maeB*, *udhA*, and *pntAB* ORFs, respectively, were transformed into *B. subtilis* strain GM1608 to generate strains GM1660, GM1661, GM1662, GM1663, GM1664, GM1665, GM1666, and GM1667, respectively. The N-terminal domain of *maeB* was amplified with primers lgO75 and lgO89 or with primers lgO75 and lgO74, providing fragments encoding the first 427 or 489 amino acids of MaeB (*maeBΔ1* or *maeBΔ2*, respectively). The longest fragment ends at the internal SphI restriction site of *maeB* (nucleotides 1369 to 1374). These fragments were inserted between the BamHI and SphI sites of vector pND23, and the resulting plasmids were transformed into strain GM1608 to give strains GM1669 and GM1670 (Table 1). Furthermore, native vector pND23 was transformed into strain GM1608 to give control strain GM1658. Complementation tests were performed with C minimal medium containing malate (1%), and overexpression was induced by addition of IPTG (final concentration, 0.5 mM).

Assay of NADP-dependent malic enzyme activity in total cell soluble extracts. Cells pellets of strains GM1668, GM1669, GM1670, GM1659, and 168CA were obtained from 2-ml aliquots of *B. subtilis* cultures growing exponentially in C minimal medium containing malate (10 g/liter). Cells were resuspended in 80 μl of Tris HCl, pH 7.4, containing 0.025 mM $MgCl_2$. Lysozyme (0.8 mg) was added, and cells were incubated at 37°C for 30 min and then centrifuged for 10 min at 4°C and 12,000 rpm. Supernatants were assayed for malic enzyme activity in the presence of NADP as described for purified proteins.

Metabolic flux ratio analysis. Cultures were grown in Erlenmeyer flasks containing 30 ml of M9 minimal medium (21) [$Na_2HPO_4 \cdot 7H_2O$ at 12.8 g/liter, KH_2PO_4 at 3 g/liter, NaCl at 0.5 g/liter, NH_4Cl at 1 g/liter, $MgSO_4$ at 1 mM, $CaCl_2$ at 0.1 mM] with either 1 g/liter [U-^{13}C]glucose and 4 g/liter naturally labeled glucose or 5 g/liter [1-^{13}C]glucose. Flasks were inoculated at 1:100 with exponentially growing precultures. Culture aliquots were harvested at an OD_{600}

of 1 to 1.2 and then hydrolyzed and derivatized as described elsewhere (35). Derivatized amino acids were then analyzed on a series 8000 gas chromatograph combined with an MD 800 mass spectrometer (Fisons Instruments, Beverly, MA). The gas chromatography-mass spectrometry-derived mass isotope distributions of proteinogenic amino acids were corrected for naturally occurring isotopes (15). The corrected mass distributions were related to the in vivo metabolic activities by previously described algebraic equations and statistical data treatment, which quantified several ratios of fluxes through converging reactions and pathways to the synthesis of five intracellular metabolites (15, 16) with the software FiatFlux (36).

RESULTS

Growth phenotypes of *B. subtilis* single and multiple mutants affected in malic enzyme-like genes. To investigate the role of each of the four genes in *B. subtilis* that putatively encode malic enzyme activity, we constructed the corresponding mutant strains and studied their growth phenotype under gluconeogenic or glycolytic conditions. Single-mutant strains for each gene, GTD102 (*maeA*), GTD110 (*malS*), GM1608 (*ytsJ*), and GM1632 (*mleA*), were first tested for growth in minimal medium containing either a gluconeogenic (malate, fumarate, or succinate plus glutamate) or a glycolytic (glucose) carbon source. The results showed that strains GTD102, GTD110, and GM1632 are able to use either a gluconeogenic carbon source or glucose as efficiently as the wild-type strain (8; data not shown). Conversely, strain GM1608 showed wild-type growth on glucose but a much slower growth rate on every gluconeogenic carbon source tested (doubling time = 108 ± 6 min versus 62 ± 4 min for the wild type on malate; Fig. 2A). These results indicated that YtsJ plays a major role in malate utilization that cannot be substituted for by the three other isoforms. The other three either have no role under these conditions or can substitute for one another.

Hence, we wondered whether a role for these genes could be revealed in a *ytsJ* background. Double-mutant strains GTD111 (*maeA malS*), GTD121 (*maeA ytsJ*), GTD122 (*ytsJ malS*), and GM1645 (*ytsJ mleA*); triple-mutant strains GTD123 (*maeA malS ytsJ*) and GM1655 (*maeA malS mleA*); and quadruple-mutant strain GM1621 (*ytsJ maeA malS mleA*) were constructed. All of these strains showed a wild-type growth rate in glucose minimal medium. Strains GTD121, GTD122, and GM1645 exhibited the same growth rate decrease as the *ytsJ* mutant strain in malate minimal medium, whereas GTD111 showed wild-type growth in this medium (data not shown). The *maeA malS mleA* triple mutation led to a moderate growth defect in malate minimal medium (doubling time = 84 ± 4 min) compared to the wild-type strain, while the quadruple mutant grew much more slowly (doubling time = 154 ± 6 min) than did the *ytsJ* single mutant (Fig. 2A). These results confirmed the major role of YtsJ in the utilization of malate for growth; they suggest that MaeA, MalS, and MleA play a role in malate utilization that is distinct from that of YtsJ and in which they can substitute for each other.

Once transported into the cells, malate can be converted into pyruvate by a malic enzyme(s) or can be utilized in the Krebs cycle by malate dehydrogenase conversion to OAA. To test whether the residual growth on malate of strain GM1621, with the four putative malic enzyme-encoding genes deleted, relied on malate dehydrogenase activity, an *mdh* mutation was introduced into GM1621, thus generating quintuple-mutant stain GM1622. The growth tests reported in Fig. 2B indicate

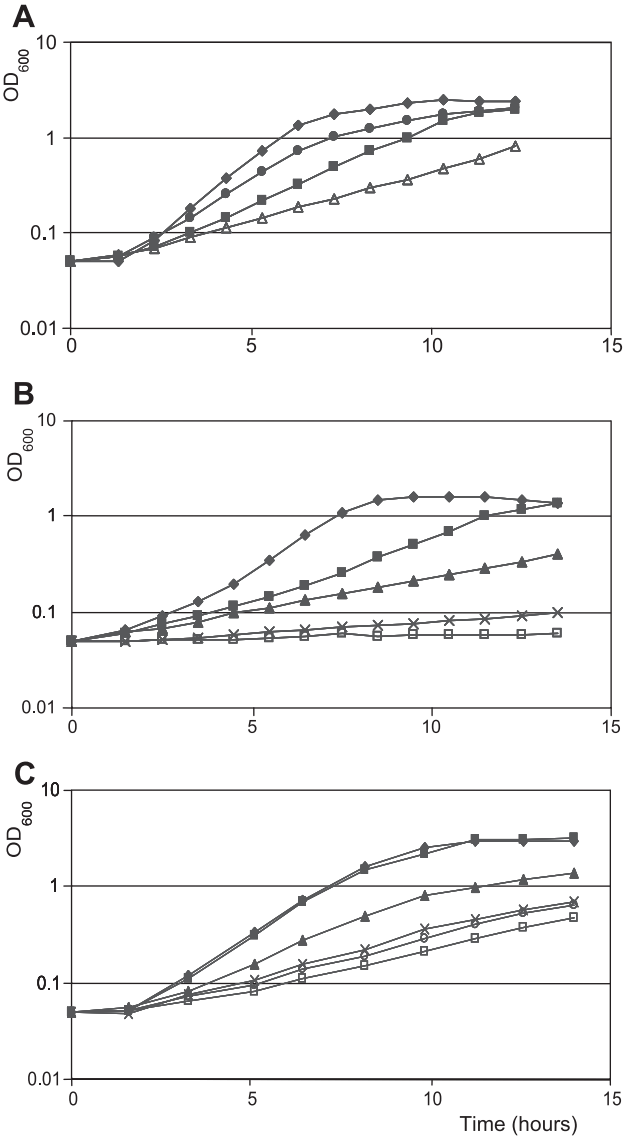

FIG. 2. Growth in malate minimal medium (A and B) and glucose minimal medium (C). *B. subtilis* strains: 168CA, ◆; GM1608 (*ytsJ*), ■; GM1621 (*ytsJ maeA malS mleA*), △; GM1655 (*maeA malS mleA*), ●; GM1614 (*mdh*), ▲; GM1615 (*ytsJ mdh*), ×; GM1622 (*ytsJ maeA malS mleA mdh*), □; GM1617 (*ytsJ maeA malS mdh*), ○. Experiments were repeated at least three times, and the results of one representative experiment are shown.

that this strain is totally unable to grow in malate minimal medium. This result confirmed that only the two pathways involving malic enzyme activity on the one hand and malate dehydrogenase activity on the other hand for malate utilization exist in *B. subtilis*. We also noted that *ytsJ* inactivation reduced the growth rate of an *mdh* mutant strain on glucose (doubling time = 196 ± 8 min versus 124 ± 4 min; Fig. 2C), even when aspartate (0.05%) was added to the medium, a supplementation that almost completely suppresses the growth defect of an *mdh* mutant strain (22). Further introductions of *maeA*, *mleA*, and *malS* mutations very slightly increased the growth defect in this medium (doubling time = 217 ± 10 min). These results

confirmed that YtsJ has a major role that is probably distinct from that of the other malic enzymes MaeA, MalS, and MleA.

Expression pattern of the *maeA*, *malS*, *ytsJ*, and *mleA* genes. In order to gain information on the physiological role of the malic enzyme-like genes, their expression pattern was investigated. The expression of *maeA*, which had been studied in our previous work, is specifically induced in the presence of malate via the two-component system MalK-MalR (formerly YufL-YufM) (8). To obtain a kinetic description of the expression of these genes, *lacZ* transcriptional reporter gene fusions were constructed and inserted into the chromosome at the *amyE* ectopic locus. Measurements of β-galactosidase activity synthesized by strain GM1600 (*amyE′*::P*ytsJ*::*lacZ*) during growth on glucose or malate minimal medium revealed fairly constant expression from the beginning of the exponential phase until the beginning of the stationary phase of growth, with a twofold higher level on malate than on glucose (Fig. 3A). By contrast, in complex medium, expression dropped at the end of the exponential phase of growth. The *malS* ORF starts 85 bp downstream of the stop codon of the *ytnP* ORF, and no putative terminator sequence can be identified within this intergenic region, suggesting cotranscription of the two ORFs. Indeed, reverse transcription-PCR experiments clearly demonstrated that mRNAs overlapping *ytnP* and *malS* are synthesized (data not shown). Furthermore, a fusion between the ca. 500 bp preceding the *malS* ORF with the *lacZ* reporter gene resulted in barely detectable β-galactosidase activity (Fig. 3B). In contrast, the fusion between the *ytnP* promoter region and *lacZ* in strain GM1602 (Fig. 3C) resulted in moderate and rather constant β-galactosidase activity at a similar level on glucose or malate minimal medium or in complex medium.

Chromosomal sequence analysis strongly suggests that *mleA* forms a bicistronic operon with *mleN*, an ORF ending 20 bp upstream of the *mleA* start codon. MleN has been shown to encode malate/Na-lactate antiporter activity (34). The P*mleN*::*lacZ* reporter fusion inserted at the *amyE* locus in strain GM1603 was poorly expressed in glucose minimal medium during the exponential phase. A similar pattern was observed on malate with ca. twofold higher maximal expression. Surprisingly, this reporter fusion was highly expressed in complex LB medium, exclusively during the transition between the exponential and stationary phases of growth (Fig. 3D).

YtsJ, MalS, and MleA exhibit malic enzyme activity, like MaeA. Our previous study had demonstrated that MaeA exhibits malic enzyme activity and preferentially utilizes NAD as a cofactor. MleA and MalS have been predicted to exhibit malolactic and malic enzyme activities, respectively (24, 34). To determine whether the three paralogous genes *ytsJ*, *malS*, and *mleA* do encode malic enzymes, the corresponding proteins were purified and their specific enzymatic activities were characterized. Vectors designed to overexpress the His-tagged YtsJ, MalS, and MleA proteins were introduced into *E. coli* strain M15(pREP4). Overexpression was induced by addition of IPTG, and the His_6-YtsJ, His_6-MalS, and His_6-MleA proteins were purified to apparent homogeneity as described in Materials and Methods (MaeA was also purified and tested again here for more accurate comparisons; the quantitative results were similar to those reported in reference 8). The capacity of these proteins to reduce NAD^+ or $NADP^+$ in the presence of malate was then assayed. These tests demonstrated

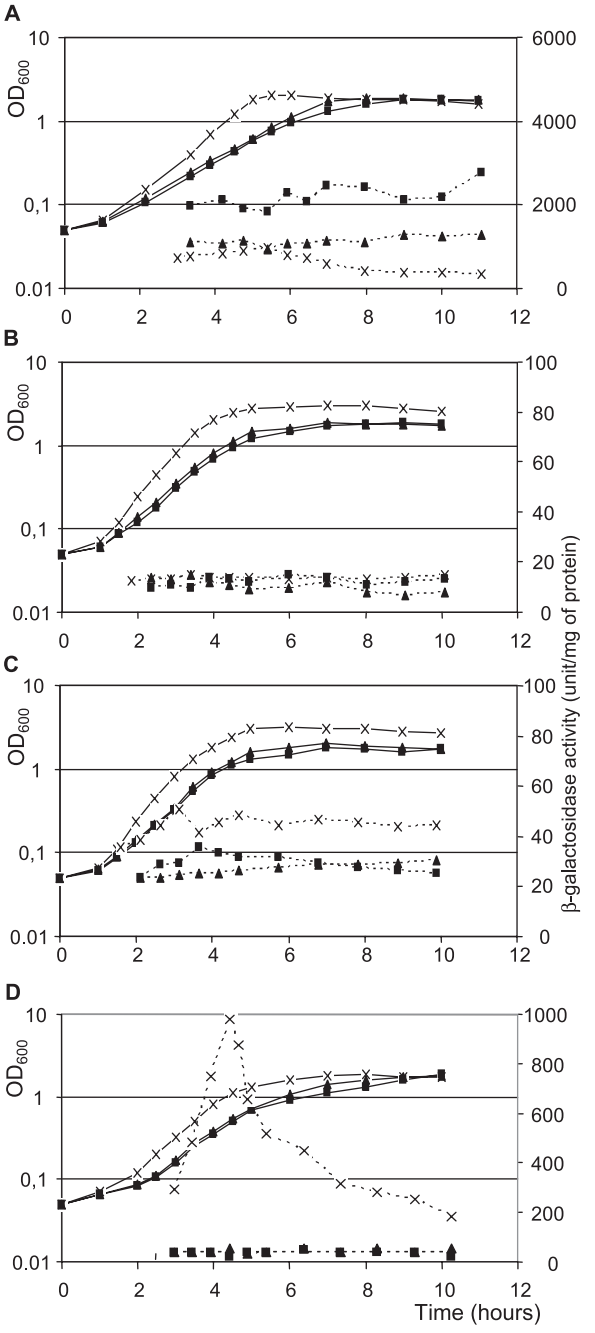

FIG. 3. Growth and reporter gene expression in different media. Media: LB, ×; glucose minimal medium, ▲; malate minimal medium, ■. Growth curves are represented by continuous lines, and β-galactosidase activities are represented by broken lines. Panels: A, GM1600 (*amyE′*::P*ytsJ*::*lacZ*); B, GM1601 (*amyE′*::*′ytnP-malS′*::*lacZ*); C, GM1602 (*amyE′*::P*ytnP*::*lacZ*); D, GM1603 (*amyE′*::P*mleN*::*lacZ*). Experiments were repeated at least three times, and the results of one representative experiment are shown.

that YtsJ, MalS, and MleA exhibit a strong ability to reduce either NAD^+ or $NADP^+$ that strictly depends on the presence of malate. To demonstrate that this reducing activity is associated with the conversion of malate into pyruvate, i.e., that the proteins exhibit malic enzyme activity and not malate dehydro-

TABLE 2. Enzymatic parameters of purified YtsJ, MleA, MalS, and MaeA

Protein	Malate[a]		NAD		NADP		OAA sp act[c] (U/mg of protein)
	K_m (mM)[b]	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (mM)[b]	k_{cat}/K_m (s^{-1} mM^{-1})	K_m (mM)[b]	k_{cat}/K_m (s^{-1} mM^{-1})	
YtsJ	1.55	35	2.8	1	0.055	73	4.60
MleA	1.56	30	1	9	ND[d]	ND	4.37
MalS	3.52	16	5.5	90	7.3	9	2.58
MaeA	3.95	19	5.3	30	6.7	8	2.03

[a] Assayed with $NADP^+$ for YtsJ and with NAD^+ for MleA, MalS, and MaeA.
[b] The mean of three independent assays is indicated. The maximal deviations from the mean were less than 10%.
[c] OAA decarboxylation activity. The mean of three independent assays is indicated. The maximal deviations from the mean were less than 10%.
[d] ND, not detected.

genase or malolactic enzyme activity, a secondary assay with lactate dehydrogenase, an enzyme specifically active on pyruvate, was coupled to the first assay. Indeed, addition of lactate dehydrogenase after the end of the first reaction (no further increase in OD_{340}) with either YtsJ, MalS, or MleA led to rapid reoxidation of NADH (data not shown). This could result only from oxidative conversion of pyruvate into lactate and thus demonstrated that pyruvate is the product of the first reaction. Kinetic parameters of YtsJ, MalS, and MleA malic enzyme activities (Table 2) were then determined as described in Materials and Methods. The K_m values of YtsJ and MleA for malate were very similar (1.55 and 1.56 mM, respectively) and about twofold lower than those of MalS and MaeA (3.52 and 3.95 mM, respectively). K_m, k_{cat}, and catalytic efficiency (k_{cat}/K_m) for NAD^+ and $NADP^+$ were calculated for the four proteins. No activity in the presence of $NADP^+$ could be detected for MleA. This malic enzyme is thus strictly NAD dependent. The k_{cat}/K_m values of MalS, 90 s^{-1} mM^{-1} for NAD and 9 s^{-1} mM^{-1} for $NADP^+$, were very close to those of MaeA (30 s^{-1} mM^{-1} and 8 s^{-1} mM^{-1}, respectively). Thus, MalS is, like MaeA, a malic enzyme with a dual specificity for NAD and NADP but with a strong preference for NAD. By contrast, the catalytic efficiency of YtsJ was much higher with $NADP^+$ than with NAD^+ (Table 2). Thus, YtsJ is also a malic enzyme with a dual specificity for NAD and NADP but with a very strong preference for NADP.

The four YtsJ, MleA, MalS, and MaeA proteins were also tested for the ability to catalyze decarboxylation of OAA (see Materials and Methods for details). As shown in Table 2, the four proteins were able to catalyze this reaction and exhibited similar specific activities. Thus, MalS, MaeA, and MalS must be assigned to the class EC 1.1.1.38 (NAD-dependent malic enzyme with OAA decarboxylation activity) and YtsJ must be assigned to the class EC 1.1.1.40 (NADP-dependent malic enzyme with OAA decarboxylation activity).

Occurrence of malic enzyme genes in bacteria. Having established that *B. subtilis* possesses four paralogous genes encoding malic enzyme activity, we wondered whether such a situation is common in other bacterial species. We therefore determined how many putative malic enzyme genes are present in each sequenced bacterial genome. According to pairwise sequence comparisons, two groups of genes could clearly be distinguished: a *ytsJ*-like group and a *maeA*-like group (Fig. S1 in the supplemental material). Our analysis revealed that most of the sequenced species contain at least one malic enzyme-like gene (Table S2 in the supplemental material). Of these, most have at least one *ytsJ*-like gene. Only a few species have three or more malic enzyme paralogs (essentially bacilli and β- or γ-proteobacteria).

A more detailed analysis of the sequenced *Firmicutes* species was then performed. Only three other *Bacillus* species or strains were found also to possess four malic enzyme genes. Four classes could be distinguished on the basis of sequence similarities and chromosomal context. *ytsJ*-like genes linked to *dnaE*, as in *B. subtilis*, form class 1. *ytsJ*-like genes linked to the *malK-malR* two-component genes (which control *maeA* induction by malate in *B. subtilis*) form class 2. *ytsJ*-like genes linked neither to *dnaE* nor to *malK-malR* form class 3. *maeA*-like genes linked to an aspartate lyase gene (as is a *ytsJ*-like gene, *mleA*, in *B. subtilis*) form class 4. The malic enzyme genes present in the *Firmicutes* species were assigned to one of these classes (Table S3 in the supplemental material).

Three main conclusions come from these analyses. (i) The bacterial species that possess at least one malic enzyme gene nearly always possess a *ytsJ*-like gene (175 of 196 species). (ii) Exchanges between the two groups of malic enzyme genes are frequently detected in members of the division *Firmicutes* (whereas a *maeA*-like gene is linked to *malK-malR* in *B. subtilis* and *B. licheniformis*, a *ytsJ*-like gene is associated with these two-component genes in other bacilli and in *Enterococcus faecalis*; an *maeA*-like gene, and not a *ytsJ*-like gene as in *B. subtilis*, is linked to an aspartate lyase gene in *B. anthracis*, *B. thuringiensis*, and *Streptococcus mutans*). (iii) Very few bacterial species (7 of 196) possess four putative malic enzyme genes, and only *B. licheniformis*, a species very closely related to *B. subtilis*, possesses four putative malic enzyme genes with the same genetic organization as in *B. subtilis*.

In vivo metabolic fluxes in malic enzyme mutants. Previous ^{13}C-based flux analyses revealed small but significant in vivo malic enzyme fluxes even during growth on glucose, when they are not required (38, 37). To identify the responsible isoform, we grew malic enzyme mutants on ^{13}C-labeled glucose. Intracellular ratios of metabolic fluxes were then quantified from the mass isotope distribution in protein-bound amino acids (15). In vivo malic enzyme activity is quantified by the molar fraction of pyruvate originating from malate, where the remaining fraction originates from pyruvate kinase (Table 3). Since the labeling pattern of malate is not directly accessible but inferred from glutamate (synthesized from 2-oxoglutarate) and aspartate (synthesized from OAA), the exchange between malate and OAA cannot be quantified. Hence, lower and upper boundaries for the fraction of pyruvate originating from malate are given which correspond to the extreme cases of

TABLE 3. Origin of metabolic intermediates during growth of *B. subtilis* malic enzyme mutants in batch cultures[a]

Parameter, SD	168CA	BFA91 (*ytsJ*)	GTD102 (*maeA*)	GTD110 (*malS*)	GTD121 (*ytsJ maeA*)	GTD122 (*ytsJ malS*)
Growth rate (1/h), ±0.05	0.43	0.39	0.52	0.40	0.47	0.46
Flux ratios						
SER through PPP, ±0.02	0.28	0.36	0.36	0.39	0.33	0.38
PEP through PPP (ub), ±0.04	0.21	0.36	0.45	0.40	0.32	0.37
OAA through TCA, ±0.02	0.50	0.47	0.50	0.44	0.50	0.45
PEP from OAA, ±0.03	0.03	0.03	0.03	0.05	0.05	0.05
PYR from MAL (lb), ±0.03	0.06	0.01	0.06	0.04	0.02	0.02
PYR from MAL (ub), ±0.05	0.11	0.02	0.11	0.08	0.03	0.03

[a] Origin of metabolic intermediates during growth of *B. subtilis* malic enzyme mutants in batch cultures grown with either a mixture of 20% U-^{13}C and naturally labeled glucose or 100% [U-^{13}C]glucose (for SER through PPP) was calculated by metabolic flux ratio analysis (15). The values presented quantify the relative contribution of one reaction or pathway to a given metabolite. Where insufficient information was available, the independently calculated upper boundary (ub) or lower boundary (lb) of the ratio is given. Standard deviations were calculated for flux ratios from experimental measurement deviations and according to Gauss' law of error propagation. SER, serine; TCA, tricarboxylic acid cycle; PYR, pyruvate; MAL, malate.

absent or complete equilibration of the malate and OAA pools, respectively (15).

In wild-type *B. subtilis* growing on glucose, between 6 and 11% of the pyruvate is typically synthesized through malic enzymes (Table 3). This contribution to pyruvate biosynthesis dropped to zero (within the limits of experimental error) in the *ytsJ* mutant, whereas knockout of the remaining isoenzymes did not affect the in vivo malic enzyme flux. Thus, YtsJ is responsible for the malate-to-pyruvate carbon flux in glucose-grown *B. subtilis*, with little or no contribution of the remaining malic enzymes. Somewhat unexpectedly, all of the malic enzyme mutants exhibited similarly increased fluxes through the pentose phosphate pathway. Since NADPH formation is one of the major functions of the pentose phosphate pathway, this increase indicates interference of the malic enzymes with NADPH metabolism.

YtsJ cannot be substituted for by the *E. coli* NADP-dependent malic enzyme for efficient growth on malate. Our phenotypic analysis showed that a *ytsJ* deletion cannot be compensated for by the presence of the three other malic enzyme-encoding genes for malate utilization. Three hypotheses, which are not mutually exclusive, can be proposed to explain this phenomenon: (i) none of the other malic enzyme genes is sufficiently expressed under the conditions of the test, (ii) the unique NADP preference of YtsJ is necessary for fully efficient malate utilization, and (iii) YtsJ has, besides the catalysis of malate decarboxylation into pyruvate, another role necessary for malate utilization that cannot be fulfilled by any of the other three malic enzymes.

To test the first hypothesis, we constructed replicative vectors designed to overexpress *malS*, *mleA*, or *maeA* under the control of the IPTG-inducible P*spac* promoter and introduced them into strain GM1608, generating GM1661, GM1662, or GM1663, respectively. As a control, the *ytsJ* gene was introduced into the same expression vector and the resulting plasmid was used to transform GM1608, creating GM1660. The growth rate of the transformed strains was measured in malate minimal medium in the presence of IPTG and compared to that of wild-type strain 168CA containing the empty replicative expression vector (GM1659). Strain GM1660 showed a roughly wild-type growth rate (Fig. 4), while GM1661 to GM1663 showed the same growth defect as the parental strain (data not shown). This indicated that overexpression of *maeA*, *malS*, or *mleA* cannot compensate for the *ytsJ* deletion and that the phenotype of the *ytsJ* mutant is indeed caused by the absence of YtsJ synthesis and not by any polar effect of the deletion.

Secondly, to test whether the NADP preference is pivotal, we supplemented the *ytsJ* mutant with a heterologous, NADP-dependent malic enzyme. For this purpose, the *E. coli maeB* gene was inserted into empty replicative expression plasmid pND23 and the resulting plasmid, pGL09, was introduced into GM1608. Tests of growth in malate minimal medium revealed no complementation of the growth defect, although, as measured in total soluble cell extracts during the exponential phase of growth, the *maeB* overexpression vector led to NADP-dependent malic enzyme activity that was 73% of that of the wild-type strain. Because MaeB is a chimeric protein with a large phosphotransacetylase-like C-terminal domain (4), similar experiments were performed with *maeB* gene derivatives with the C-terminal domain deleted (two different endpoints were considered; see Materials and Methods for details). These different constructions gave rise to 60 to 80% of the wild-type level of NADP-dependent malic enzyme activity but were unable to complement the growth defect of the *ytsJ* mutant strain into which they were introduced (Fig. 4).

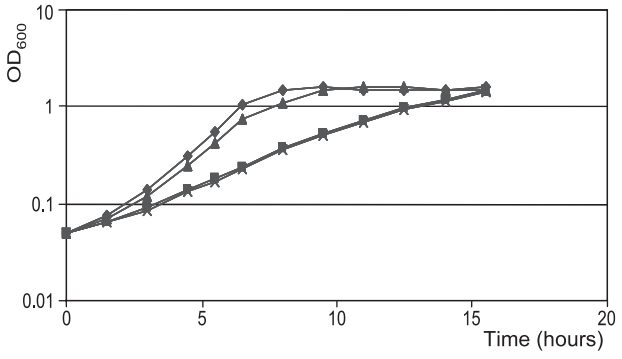

FIG. 4. Growth in malate minimal medium. Strains: GM1659 (wild type/pND23), ◆; GM1658 (Δ*ytsJ*/pND23), ■; GM1660 [Δ*ytsJ*/pGL04 (*ytsJ*)], ▲; GM1670 [Δ*ytsJ*/pGL17 (*maeB*)], ×. Experiments were repeated at least three times, and the results of one representative experiment are shown.

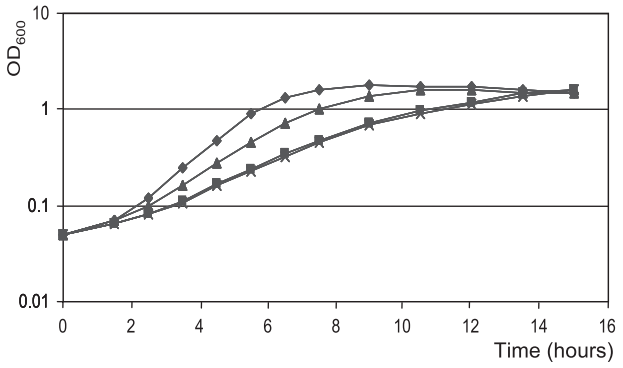

FIG. 5. Growth in malate minimal medium. Strains: GM1659 (wild type/pND23), ◆; GM1658 (Δ*ytsJ*/pND23), ■; GM1666 [Δ*ytsJ*/pGL10 (*udhA*)], ▲; GM1667 [Δ*ytsJ*/pGL11 (*pntAB*)], ×. Experiments were repeated at least three times, and the results of one representative experiment are shown.

Overexpression of the *E. coli* UdhA transhydrogenase partially suppresses the growth defect of a *B. subtilis ytsJ* mutant strain. The above results did not indicate an important role for malic enzyme-produced NADPH per se but left open a potential contribution of YtsJ to the maintenance of the NADPH-NADH balance. In the *B. subtilis* genome, no putative transhydrogenase-encoding gene has been identified nor is any other enzymatic system(s) known that could fulfill a similar function, i.e., interconversion of NADH and NADPH. To test this hypothesis, the two *E. coli* transhydrogenases, PntAB and UdhA, that catalyze the production of NADPH from NADH and the opposite reaction, respectively (29), were overexpressed from pND23 in the *ytsJ* mutant GM1608. While *pntAB* overexpression did not affect the growth rate of the *ytsJ* mutant strain, overexpression of *udhA* (GM1666) partially suppressed the growth defect on malate (Fig. 5). On malate, the doubling times of the wild type, the *ytsJ* mutant, and its UdhA-overexpressing derivative were 65, 130, and 105 (± 6) min, respectively. Overexpression of *udhA* partially relieved the metabolic defect caused by the *ytsJ* knockout but had no effect on the growth rate of a wild-type strain (data not shown).

DISCUSSION

The present study demonstrates that *B. subtilis* indeed possesses four paralogous genes encoding malic enzyme activities. According to our enzymatic tests of the purified proteins, MalS and MaeA belong to class EC 1.1.1.38, which groups malic enzymes that have a dual specificity for NAD and NADP as a cofactor but have a strong preference for NAD and are able to decarboxylate OAA. YtsJ falls into the EC 1.1.1.40 category as a malic enzyme capable of preferentially NADP-coupled malate and OAA decarboxylation. The NADP dependency, however, is not as strict as in most of the enzymes of this class. MleA is a strictly NAD-dependent malic enzyme that, in contrast to the enzymes of class EC 1.1.1.39, can decarboxylate OAA; by this argument, it should be in the EC 1.1.1.38 category.

All of the malic enzymes from eubacteria and eukaryotes characterized to date belong to the same protein family, together with the bacterial malolactic enzymes. Numerous biochemical and structural studies of eukaryotic malic enzymes (reviewed in reference 4) have allowed not only description of the catalytic mechanism but also identification of the residues involved in substrate and metal ion binding, as well as in catalysis. These residues are highly conserved in the four malic enzymes of *B. subtilis* (Fig. S1 in the supplemental material). Such extensive studies have not yet allowed unequivocal identification of the residues involved in preferential binding of NAD or NADP. However a careful examination of the different biochemical and structural data available led Chang and Tong (4) to identify two residues (Ser346 and Lys362 in the pigeon NADP-dependent malic enzyme) that would be directly responsible for NADP specificity. It is interesting that these residues are conserved in YtsJ but not in the other three malic enzymes of *B. subtilis* (Fig. S1 in the supplemental material), in agreement with the cofactor preferences of these enzymes characterized in this study.

What is the physiological function of malic enzymes? While expressed during growth on malate, they are not strictly necessary because malate can also be utilized via OAA conversion to PEP by using malate dehydrogenase and PEP-carboxykinase (PckA). Indeed, an *E. coli* double-mutant strain with both malic enzymes deleted is clearly but moderately affected in growth on malate (33). Instead, malic enzyme activity is assumed to increase the efficiency of malate utilization by contributing to a correct balance among the three key metabolites OAA, PEP, and pyruvate and, in the case of NADP-dependent malic enzyme activity, by producing the main source of reducing power for anabolism, NADPH (30).

Our phenotypic analysis clearly established that YtsJ plays an important role in efficient growth on malate that cannot be fulfilled by any of the other three malic enzymes, even when these are artificially overexpressed. This indicates distinct and synergistic roles for YtsJ compared to MalS, YwkA, and MleA (which can substitute for each other; Fig. 2). The four K_m values for malate were very similar (Table 2) and thus can hardly explain these distinct roles. Similarly, the catalytic efficiencies (with NADP for YtsJ and with NAD for MaeA and MalS) were in the same range. Since YtsJ was the only NADP-dependent enzyme, we reasoned that its singular physiological role could be linked to this property. Overexpression of NADP-dependent MaeB from *E. coli*, however, did not complement the *ytsJ B. subtilis* mutant phenotype, despite wild-type-like NADP-dependent malic enzyme activity synthesized in the transformed strain. This result suggests that the role of YtsJ during growth on malate relies not only on its NADP-dependent malic enzyme activity. Nevertheless, we cannot exclude the possibility that the absence of complementation is due to kinetic parameters and/or to allosteric effectors of MaeB that are significantly different from those of YtsJ.

Much to our surprise, overexpression of the soluble *E. coli* transhydrogenase UdhA partially suppressed the growth defect of the *ytsJ* knockout on malate. The sole function of this enzyme is fully reversible conversion of NADPH and NADH. During growth of *E. coli* on glucose, the physiological role of UdhA is conversion of NADPH to NADH, while the membrane-associated transhydrogenase PntAB catalyzes the opposite reaction (29). During growth of the *ytsJ* mutant on malate, however, we expect massive production of NADH because all of the malate must be catabolized through either NADH-producing malate dehydrogenase or the NADH-producing

malic enzyme and pyruvate dehydrogenase. Thus, it is not unreasonable to hypothesize that the cofactor concentrations are different from those during growth on glucose, such that UdhA could indeed catalyze NADPH production from NADH. The fact that *pntAB* overexpression does not complement the *ytsJ* phenotype—although it should, on the basis of its function in *E. coli*—might be related to its incorrect folding or the inability to form an active membrane-associated complex upon heterologous expression.

Since the partial complementation of UdhA overexpression can only be explained by its known involvement in cofactor balancing, we assume that at least one important physiological role of YtsJ is related to NADPH metabolism. This hypothesis, however, remains to be verified by cofactor concentration and detailed flux analyses. The current data suggest that YtsJ might have another function that is independent from the NADP-dependent conversion of malate to pyruvate, and this would explain why UdhA complements only partially. This additional function is only necessary under conditions with high malate-to-pyruvate fluxes, i.e., growth on malate, fumarate, or succinate, or in *mdh* mutants but not during growth on glucose.

The large majority of sequenced bacterial species have at least one malic enzyme-like gene, and most of these species have at least one *ytsJ*-like gene (Table S2 in the supplemental material). This higher conservation of *ytsJ*, which is particularly clear among bacilli, staphylococci, and clostridia, might indicate that a YtsJ-like enzyme also has a major physiological role in species other than *B. subtilis*. A second *ytsJ* homolog is present in bacilli other than *B. subtilis* and *B. licheniformis* which is linked to *malK-malR* two-component genes, whereas a *maeA* gene is associated with *malK-malR* in *B. subtilis* and *B. licheniformis* (Table S3 in the supplemental material). This exchange between the two different groups of malic enzyme genes suggests that a *ytsJ*-like gene can fulfill the specific function associated with MalK-MalR (while a *maeA*-like gene is unable to back up the major function of *ytsJ* in *B. subtilis*). Finally, *B. anthracis*, *B. subtilis*, and *B. licheniformis* appear to be singular in having four malic enzyme paralogs (together with only *Streptomyces avermitilis*, *Colwellia psychrerythraea*, and *Photobacterium profundum* of more than 300 species considered) but precise selective advantages conferred by this property and ensuring the conservation of this multigenic family remain to be clarified.

Whereas *maeA* transcription is specifically induced in the presence of malate in the growth medium (8), the *ytsJ* gene was transcribed at a high level under all of the conditions tested. This transcriptional pattern of *ytsJ* is in accordance with the major physiological role of the corresponding enzyme for efficient utilization of different C_4 dicarboxylates, and not only malate, for growth but also more generally in central carbon metabolism (Table 3). On the contrary, the very weak transcription of *malS* or *mleA* in glucose or malate minimal medium suggests that they could have a physiological significance under specific conditions not explored in this study. The spectacular increase in *mleA* transcription in complex medium only during the exponential phase of growth could illustrate such specific conditions. The identification of the parameter responsible for high *mleA* transcription will give insights into the physiological conditions under which *mleA* is important. Our enzymatic tests showed that, in contrast to what has been proposed (34), MleA is not a malolactic enzyme; actually, a malolactic enzyme converts malate into lactate without release of pyruvate. It should also be noted that the two bacterial malolactic enzymes demonstrated enzymatically have a sequence much more similar to that of MaeA or MalS than to that of MleA or YtsJ. However, MleA could have a particularly significant role in acting together with lactate dehydrogenase to catalyze a coupled reaction equivalent to a malolactic enzyme reaction. This could explain its cotranscription with *mleN*, which encodes a malate-lactate antiporter (34).

In conclusion, *B. subtilis* possesses four paralogous genes encoding malic enzyme activity that are not physiologically equivalent. On the contrary, YtsJ exhibits a singular specificity for NADP as a cofactor and plays a major physiological role for which the three other malic enzyme cannot compensate. Our results indicate that this major role would be related to NADPH metabolism, but complementary experiments are necessary to determine its precise contribution to this metabolism.

ACKNOWLEDGMENTS

We thank Dusko Ehrlich, Linc Sonenshein, and Patrick Stragier for kindly providing strain BFA91, strain SJB238, and plasmid pEC23, respectively. We thank Dominique Le Coq and Colin Tinsley for helpful comments on the manuscript and Claude Gaillardin, Josef Deutscher, and Jean-Marie Beckerich for valuable discussions and continuous encouragement.

Guillaume Lerondel is on a fellowship from the French Ministère de la Recherche et de la Technologie. Nicola Zamboni was supported by DSM Nutritional Products. Thierry Doan was and Stéphane Aymerich is on the staff at INRA. This work was supported by EU Quality of Life and Management of Living Resources program grant QL GZ-CT-1999-O1455.

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